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PROTEIN EXPRESSION IS ALTERED DURING SPONTANEOUS SLEEP IN AGED SPRAGUE-DAWLEY RATS

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Abstract

Age-related changes in brain function include those affecting learning, memory, and sleep-wakefulness. Sleep-wakefulness is an essential behavior that results from the interaction of multiple brain regions, peptides and neurotransmitters. The biological function(s) of sleep, however, remains unknown, due to a paucity of information available at the cellular level. Aged rats exhibit alterations in the circadian and homeostatic influences associated with sleep-wake regulation. We recently showed that alterations in cortical profiles occur after timed bouts of spontaneous sleep in young rats. Examination of the cellular response to sleep-wake in old rats may thus provide insight(s) into the biological function(s) of sleep. To test this hypothesis, we monitored cortical profiles in the frontal cortex of young and old Sprague-Dawley rats after timed bouts of spontaneous sleep-wake behavior. Proteins were separated by two-dimensional electrophoresis (2-DE), visualized by fluorescent staining, imaged, and analyzed as a function of behavioral state and age. Old rats showed a 6-fold increase in total protein expression, independent of the behavioral state at sacrifice. When analyzed according to age and behavioral state, there was a decrease (~46%) in the number of phospho-spots present during SWS in aged animals. SWS-associated spots present only in old animals were associated with multiple functions including vesicular transport, cell signaling, oxidation state, cytoskeletal support, and energy metabolism. These data suggest that the intracellular response to the signaling associated with spontaneous sleep is affected by age and is consistent with the idea that the ability of sleep to fulfill its' function(s) may become diminished with age.

Keywords

two-dimensional electrophoresis (2DE); mass spectrometry; sleep-associated proteins; spontaneous sleep bouts; aging

1. Introduction

Aging is associated with a generalized and progressive reduction in the body's ability to respond to stress that is thought to arise from damage to intracellular organelles and macromolecules (i.e., DNA, proteins, and lipids) as a result of the accumulation of reactive

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oxygen and nitrogen species, ROS and RNS, respectively; (Balaban et al., 2005; Calabrese et al., 2006; Joseph et al., 2005). The covalent binding of these species affects the activity of a host of proteins, thereby affecting protein function and turnover (Stadtman, 1990; Stadtman, 1992) and has been documented in bacteria (Cabiscol et al., 2000; Nystrom, 2002), fly (Sohal, 2002), and rat (Butterfield et al., 1999; Kanski et al., 2003) models of aging. In the rat, the functional categories of covalently modified proteins are similar in muscle and brain and include proteins involved in biosynthesis, energy production, cytoskeletal dynamics and signal transduction (Butterfield et al., 1999; Kanski et al., 2003; Poon et al., 2004a; Poon et al., 2004b; Poon et al., 2006).

The brain is particularly susceptible to oxidative stress, with high levels of unsaturated fatty acids, iron/ascorbate, and oxygen consumption and relatively low levels of proteins involved in antioxidant defense (Floyd, 1999; Poon et al., 2004a; Poon et al., 2004b; Poon et al., 2006). The severity of symptoms can vary. Mitochondrial dysfunction, for example, has been linked to neurodegenerative disorders like Alzheimer's disease, Parkinson's disease, and Huntington's disease (Genova et al., 2004), while "normal" aging is associated with alterations in a broad spectrum of brain functions that include learning, memory, and sleep (Calabrese et al., 2006).

Sleep is a relatively simple behavior with complex anatomical and physiological underpinnings. The existing data indicate that sleep is an essential behavior, regulated by a combination of circadian and homeostatic influences (Kryger et al., 2000). The biological function(s) remains unknown, though there is general agreement that sleep serves a restorative function(s). Key to the elucidation of the biological function (s) is an understanding of how signals associated with sleep impact the cellular milieu. Early intracellular studies from the 1970's suggested that sleep may replenish proteins. Studies using rapid eye movement (REM) sleep deprivation to investigate the function of REM sleep showed that protein synthesis occurs during REM sleep, suggesting a restorative function of sleep (Bobillier et al., 1971; Drucker-Colin et al., 1979). A subsequent study that examined ^{14}C-leucine absorption into cerebral protein showed that protein synthesis increased during SWS compared with REM sleep and waking (Ramm and Smith, 1990). These early and seemingly conflicting results may reflect the sensitivity of the methods employed or the relatively large areas tested. Sleep research has greatly benefited from recent technological advances in high throughput analyses of mRNA and protein profiles (Basheer et al., 2005; Cirelli et al., 2004; Terao et al., 2006; Vazquez et al., 2008). The results of these recent studies identified proteins/mRNAs associated with ATP generation/storage, the oxidation reduction state and cytoskeletal metabolism, suggesting roles for sleep in the maintenance of energy metabolism, re-dox state and synaptic plasticity in young rats. There is thus considerable overlap in the functional categories associated with sleep and those affected by aging.

Aging negatively impacts sleep-wake behavior in clinically compromised and otherwise healthy elderly humans (Avidan, 2005; Montgomery and Dennis, 2004; Prinz, 1995). In aged humans, there is a decrease in "deep" (or "restorative") sleep and an increase in "light" or early stages of sleep that leads to more frequent awakenings and changes in sleep timing (Bliwise, 1993; Prinz, 1995). In addition, the ability to compensate for sleep loss after prolonged waking is diminished (Bonnet and Rosa, 1987; Carskadon and Dement, 1985). Other experimental models including rodents (Shiromani et al., 2000), flies (Cirelli, 2006), and zebrafish (Zhdanova et al., 2008) also exhibit age-related alterations in sleep. In aged rodents (Shiromani et al., 2000), the inability to compensate for sleep loss is accompanied by reductions in protein/mRNA (Cunha et al., 1995; Sperlagh et al., 1997) and/or alterations in protein function (Basheer et al., 2005) and protein profiles (Pawlyk et al., 2007). Taken together, these results provide evidence that the intracellular response to sleep is diminished during aging in brain regions associated with sleep-wakefulness under both normal and stressful conditions.

Based on this data, we hypothesized that aging would have pronounced effects on protein profiles across spontaneous sleep-wake bouts. In addition, we hypothesized that the functional categories of proteins affected by aging would be related to those associated with sleep in young animals. To test this hypothesis, we compared protein profiles from the frontal cortex of young and old rats following timed bouts of spontaneous sleep. Proteins separated by two-dimensional electrophoresis were sequentially stained with ProQ Diamond and SYPRO Ruby to visualize phosphorylated and total protein, respectively. Spots showing SWS- and age- related were isolated, eluted, and identified by a combination of mass spectroscopy analyses

2. Results

2.1 Sleep and wake parameters across age

Old rats had decreased percentages of slow wave sleep (SWS; t= 3.87, df= 6, *p=0.004), total sleep time (%TST; t= 3.54, df= 6, *p=0.006) and an increased percentage of waking (W; t= −3.56, df= 6, *p=0.005) during the lights-on period only (Figure 1A, Figure 1B), consistent with previous results (Van Gool and Mirmiran, 1983).

2.2 Protein expression is increased in aged rats

Master gel images from young and old animals were generated and analyzed for SYPRO Ruby (Figure 2B) and Pro-Q Diamond (Figure 2C) stains. A total of 822 protein spots (Figure 2A, left) were distinguished in the SYPRO Ruby Master image, 68% of which were present in both age groups. Only 4% of the spots (n=36) were unique to young animals. The number of SYPRO Ruby spots present only in aged animals, however, accounted for 28% (n=231) of the total number of spots visualized, a 7-fold increase over that of young rats. A total of 102 phosphorylated spots was visualized using Pro-Q Diamond (Figure 2A, right), with approximately 51% of the phosphorylated proteins present in both W and SWS states (n=52). The number of phosphorylated spots, however, did not vary between young (n=26) and old rats (n= 24; Figure 2A).

2.3 Protein expression is altered by behavioral state in young and aged rats

Table 1 (left panel) shows the analysis of SYPRO Ruby spots as a function of age and behavioral state. In young rats, ~3% were present in both W and SWS states (n=18). State-related expression also accounted for nearly 3% of the total visualized in young rats (W, ~1%; SWS, ~2%). In old animals, approximately 18% (n=142) were visualized in both behavioral states, while about 12% of the spots showed state-related expression. Of the state-related spots visualized in aged rats, ~4% spots (n=30) were W-related and ~8% spots (n=142) were unique to SWS. Thus, there was an increase in the number of SYPRO Ruby spots in aged rats that was independent of behavioral state. The Pro-Q Diamond staining showed that there was no change in the number of phosphorylated spots visualized in either age group (Figure 2A). However, a decrease in the number of phosphorylated spots during SWS was evident in old rats (Table 1, right panel). In young rats, phosphorylated spots accounted for ~27% of the total spots visualized, compared to only 12% in aged rats. The marked decrease in the number of phosphorylated proteins in aged rats during sleep (~56%) thus provides evidence that cellular activities associated with SWS are altered as a function of age.

2.4 Identification of SWS-associated spots in aged animals

SWS-specific spots (n=5) from aged animals were excised from Gel Code Blue stained gels, eluted and identified by MS/MS analyses (Figure 3A). A total of 42 proteins were identified from these spots with high confidence from old rats sacrificed during SWS (Figure 3A, arrows). A pie chart shows the functional categories associated with the identified proteins (Figure 3B). Table 2 is a complete listing of all proteins identified from the MS analyses. Proteins identified

included those associated with vesicular transport (n=9), cell signaling/transduction (n=6), oxidation state (n=5), cytoskeletal support (n=4), energy metabolism (n=4), mitochondrial (n=3), nucleic acid (n=3), and amino acid metabolism (n=3), neurotransmitter biosynthesis (n=2) and proteolysis (n=2). Vesicular transport proteins included ADP-ribosylation factor 1, ADP-ribosylation factor-like protein 8B, Rab11B, Rab 3A, Rab 2A, Rab18, tumor protein D52-like protein, alpha-synuclein, and vesicle-associated membrane protein (Becher et al., 1999; Cao et al., 2006; Haraguchi et al., 2006; Khvotchev et al., 2003; Malagon et al., 2005; Mattson et al., 2002; Sudhof, 2000; Suzuki et al., 2001). Proteins associated with signal transduction included phosphatidylethanolamine-binding protein 1, Rap1B, olfactory marker protein, visinin-like protein 1, translation initiation factor eIF-5A, and dimethylarginine dimethylaminohydrolase 2 (Enwere et al., 2004; George et al., 2006; Hasegawa et al., 2006; Poon et al., 2006; Ribeiro-Neto et al., 2004). Proteins associated with maintenance of the oxidation reduction state included glutathione S-transferase Yb-3, superoxide dismutase, peroxiredoxins 1 and 2, and hemoglobin subunit alpha-1/2. Four of the identified proteins are involved in cytoskeletal support (i.e. profilin-2, platelet-activating factor acetylhydrolase 1b [subunit beta], myelin basic protein and thy-1 membrane glycoprotein; (Mastronardi and Moscarello, 2005; Webster et al., 1999; Witke, 2004; Yan et al., 2003). Proteins associated with the generation of ATP were identified from both cytosolic (i.e. phosphoglycerate mutase 1, 6-phosphogluconolactonase, lactoylglutathione lyase; (Chesler, 2003; Delarue et al., 2007; Ikemoto and Ueda, 2003; Thornalley, 1990) and mitochondrial (i.e. prohibitin, NADH dehydrogenase Fe-S protein 3, S1 chain; (Kushnareva et al., 2002; Mihara and Omura, 1996; Vessal et al., 2006) compartments. Proteins linked to nucleic acid metabolism (i.e. guanylate kinase, histone H2A, histone H4; (Funke et al., 2005; Kim et al., 2001), amino acid metabolism (i.e. dihydropteridine reductase, phosphoserine phosphatase, histidine triad nucleotide-binding protein 1;(de Koning, 2006; Kitzerow and Henrich, 2001; Thony et al., 2000) and neurotransmitter biosynthesis (protein kinase C inhibitor protein 1, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase; (Ichimura et al., 1987; Toker et al., 1992) were also identified. Finally, proteins associated with degradation via proteolysis or ubiquitination mechanisms were also identified (i.e. proteasome 28 subunit, alpha; signalosome subunit 8; (Lykke-Andersen and Wei, 2003; Rechsteiner and Hill, 2005).

3. Discussion

Aging is associated with sleep disruptions that diminish the quality of life in the elderly (Phillips and Ancoli-Israel, 2001). Decreased frontal cortex activity during sleep has been correlated with age-associated brain atrophy, synaptic degeneration, neurochemical alterations, and reduced blood flow (Cabeza et al., 2002). We examined putative links between aging and sleep at the protein level. While changes in regional protein expression occurred within minutes of spontaneous sleep-wake bouts in both young and aged animals, our data shows that the cellular response to sleep is dramatically altered by age. The marked increase in the number of SYPRO Ruby spots in old compared to young animals, independent of sleep-wake behavior is consistent with the progressive accumulation of oxidized proteins that accompany aging in healthy as well as those with neurodegenerative diseases (Kanski et al., 2003; Poon et al., 2006). The age-related decrease in phosphoprotein expression during SWS is consistent with a decrease in cellular activities and the dysregulation of cellular activity that occurs following sleep deprivation (Basheer et al., 2005). Further, the SWS-related proteins identified in old rats (Table 2) are associated with the same functional categories as those identified in young animals across spontaneous sleep-wake by both mRNA (Cirelli et al., 2004) and protein analyses (Vazquez et al., 2008). One such protein, superoxide dismutase, is critical in protecting against ROS (Calabrese et al., 2006) and has also been implicated in the disruption of sleep regulation in young animals (Ramanathan et al., 2002) and humans (Christou et al., 2003). In addition, members of the Rab family, profilin, and ADP-ribosylation factors are essential for the maintenance of synaptic plasticity (Witke, 2004). Further, alpha-synuclein, is also critical for

synaptic integrity (Hashimoto et al., 2004). Alpha-synuclein abnormalities results in Lewy body inclusions observed in Parkinson's disease (PD), and have also been observed in patients with REM sleep behavior disorder (RBD; (Turner et al., 2000). Our results are thus consistent with an age-related dysfunctions in antioxidant potential (Sivonova et al., 2007), in macromolecular assemblies required for nucleic acid and protein metabolism (Piwien-Pilipuk et al., 2002), neurotransmitter synthesis (Cruz-Muros et al., 2007) and protein degradation (Dahlmann, 2007). While further studies are needed to provide more detailed insights concerning the role of aging on sleep, our results reveal important clues to understanding both age-associated alterations in sleep architecture and the cellular underpinnings of sleep.

4. Experimental Procedure

4.1. Experimental design and analysis of behavioral state

All animal procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, 7th ed (Council, 1996). Young (~3 mo.; n=9) and aged (~22 mo.; n=8) male Sprague-Dawley rats (250–500g) were implanted with electroencephalographic (EEG) and electomyographic (EMG) to monitor brain and muscle activities, respectively. A catheter was also inserted into the femoral vein to permit sacrifice without contacting the animals. All rats were individually housed (12:12; lights-on, 8 am) and habituated to the recording apparatus with free access to food and water. Spontaneous sleep-wake activity was recorded for 48 h prior to beginning the experimental protocol. Wake (W), slow wave sleep (SWS), and rapid eye movement (REM) sleep states were captured digitally, stored to disk and scored manually. W was defined by the presence of low amplitude, high frequency EEG waves with high EMG activity. SWS was identified by high amplitude slow waves in conjunction with a lower level of EMG activity compared to waking. REM sleep was characterized by the presences of rhythmic theta EEG waves and little or no EMG activity. Sleep-wake states were scored manually in 10 sec epochs. The percents of W, SWS, REM and total sleep time (%TST; the sum of SWS plus REM sleep percentages) were determined. Rats were sacrificed following timed bouts of spontaneous SWS and W as previously described (Greco et al., 1999; Vazquez et al., 2008). Briefly, rats were sacrificed after 10 minutes of continuous waking or following 10 minutes of continuous SWS between 3–4 pm. This time frame is during the lights-on period, the rats' normal sleep time and corresponds to the latter third of the lights-on phase. All rats were euthanized with an overdose of pentobarbital (200 mg/kg, i.v.) injected into a femoral vein catheter.

4.2. Protein sample lysis, separation and staining

Bilateral frontal cortex tissue (5.2 mm - 4.2 mm anterior to Bregma) (Paxinos and Watson, 1982) was homogenized in buffer containing 7M urea, 2M thiourea, 2% CHAPS, 2% ASB-14, 20 mM Tris, 1% DTT (wt/vol), 0.2% 3/10 ampholytes (vol/vol), and 1% protease inhibitors as described (Vazquez et al., 2008). Briefly, protein lysates (100ug/gel) were first separated according to net charge on IPG strips (pH 3–10 NL; 11 cm). In the second dimension, proteins were separated by molecular mass using Tris-HCL gels (11cm) with a polyacrylamide gradient of 4–15%. Each gel was sequentially stained, first with Pro-Q Diamond (Invitrogen, Carlsbad, CA), followed by SYPRO Ruby (Invitrogen) according to the respective manufacturer's instructions. Pro-Q Diamond, a fluorescent stain that binds to phosphorylated proteins, was used as a marker of regional cellular activity (Graves and Haystead, 2002; Hunter, 1995; Krebs, 1994; Sun and Tonks, 1994). SYPRO Ruby is a fluorescent stain used to visualize total protein expression patterns. Each lysate was separated and analyzed in duplicate. The use of commercially available pI strips, polyacrylamide gels, high through-put power supplies/ equipment that permit the simultaneous separation of up to 12 samples, and sensitive fluorescent stains results in improved between-gel reproducibility for spot detection and resolution (Boonjakuakul et al., 2007; Zhan and Desiderio, 2003a; Zhan and Desiderio,

2003b). To obtain enough protein to ensure accurate MS identification of spots and to verify the results from the combined analyses of individual samples (above), a second series of 2DE separations were performed on lysates (1 mg) that were pooled according to behavioral state prior to separation (1st dimension, pH 3–10 NL strips, 17 cm; 2cd dimension, 10% polyacrylamide Tris-HCl gels). These gels were stained with GelCode Blue® according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL). All stained gels were digitally captured using a high resolution scanner (Molecular Imager FX, BioRad). Thus, for these analyses, two complimentary approaches were used to determine age- and state- related expression. In the first analyses, state-related expression from individual profiles were separated on "midi" analytical gels and visualized with a combination of fluorescent stains. In the second set of analyses, individual samples were pooled, separated by 2DE using a giant gel format (17 × 17 cm), and stained with GelCode Blue. Spots that were unique-SWS in old rats were identified by subtractive analysis of the Master gel image generated from the comparison of these gels. Agreement between images generated from individual and pooled sample sets (see below) with regard to isoelectric point, mass, and state-related expression was thus a prerequisite for spot picking and subsequent MS analyses.

4.3. Image analyses

Protein spots were analyzed using PDQuest 2-D Image Analysis Software (BioRad) and have been described in detail (Vazquez et al., 2008). Duplicate samples from each animal (i.e., a total of 34 gels from 17 rats) were analyzed to reduce spot detection errors. Spots were identified as the sum of the intensities of the image pixels within a boundary, where spot height or peak value on an *x* and *y* axis were measured (ODs or counts/image units^2) and fitted to the scanned spots using Gaussian curves. Gaussian modeling facilitates the identification of overlapping spots, spots in gel streaks, and multiple spots in dense clusters. Errors detected by the automated matching of spots were manually inspected. Spot intensities of equal to or less than 4-fold were considered artifacts and/or background staining. Data was analyzed first as a function of age, independent of behavioral state. The second analysis examined protein expression as a function of age and behavioral state at sacrifice. In each analysis, samples were grouped according to age and/or behavioral state, spot matched, and normalized to one another to create a gel composite. Gel composites were used to generate a Master image for each stain and experimental condition. Averaging parameters (criteria to assist with group comparisons) across experimental groups were set to equal to or greater than 70% consistent with instrument specifications (Zhan and Desiderio, 2003a; Zhan and Desiderio, 2003b). Master gel images for each stain were subsequently generated by combining all spots identified in all gels across all experimental groups. Spots from each behavioral state were compared and matched to the appropriate Master image (i.e., either ProQ Diamond or SYPRO Ruby). Qualitative analysis sets were generated by subtractive (Boolean) analyses to identify spots: 1) common to all conditions as a function of age *and* behavioral state; 2) present *only* as a function of age; and 3) unique to either waking *or* sleep state as a function of age.

4.4. Mass spectrometry and protein identification

Spots selected for protein identification were excised from GelCode Blue® stained gels followed by in-gel tryptic digestion and peptide extraction as previously published (Boonjakuakul et al., 2007). Protein identifications were established by HPLC/ESI/MS/MS analysis of the resulting peptide extracts. Liquid chromatographic separations were performed on an Ultimate Capillary HPLC System (Dionex/LC Packings, Sunnyvale, CA) equipped with a PepMap (Dionex/LC Packings) trap column and a reversed phase C18 nano-column (75 m i.d. × 150 mm packed in-house with Jupiter Proteo C12 end-capped resin, 90 Å pore size, 4 m particle size) and a Famos Micro autosampler. An aliquot of peptide extract (3–4 μL) was loaded onto the trap column with loading solvent (0.1% formic acid) at a flow rate of 20 μL/min. The trap column was washed with the loading solvent for 3 minutes prior to switching it

in line with the reversed phase nano-column. The nano-column and elution buffers were maintained at ambient temperature and the mobile phase flow rate was 325 nL/min. The nano-column was equilibrated with 2% B for 20 min prior to sample injection (Solvent A: 2% acetonitrile/0.1% formic acid; Solvent B: 80% acetonitrile/0.08% formic acid). Peptide separation was accomplished using a binary gradient which consisted of a 5 min isocratic wash at 2% B followed by a linear gradient of 2%–50% B over 45 min, and concluded with a column cleanup step of 95% B for 7 min. The column effluent flowed directly into a nanoelectrospray ion source (Protana) on a QSTAR XL quadrupole/quadrupole/time-of-flight (QqTOF) mass spectrometer (Applied Biosystems/MDS SCIEX). Protein identification was accomplished by isolating sequentially eluting peptide populations with a single mass-to-charge ratio (*m*/*z*), within the mass spectrometer, fragmenting this population, and measuring the masses of the peptide fragment ions.

Mascot™ (version 2.2.04, Matrix Sciences) software was used for peak detection, creating mass peak lists and performing database searches. The experimentally determined peptide fragment ion masses were used to search a theoretical fragment ion mass database generated by *in silico* digestion and fragmentation of all *Rodentia* proteins in the SwissProt database (created 04-23-2009, 462764 sequences, 163773385 residues). Search parameters were employed with trypsin as the enzyme specificity (maximum number of missed cleavages set at 2), carbamidomethylation as a fixed modification of cysteine residues and variable modifications of deamidated Asn and Gln; pyro-Glu; and oxidized Met. The peptide and fragment ion mass tolerances were ± 150 ppm and ± 0.15 Da, respectively. Mascot peptide acceptance criteria required individual ion scores to be > 33 which indicated protein identity or extensive homology at a p value < 0.05 (95% confidence level). The MS/MS spectra for all protein identifications based on ≤ 4 peptide identifications were manually verified. The Mascot search engine automatically performed a decoy database search to determine the false discovery rate (FDR).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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A

State	Lights On		Lights Off	
	Young	Aged	Young	Aged
% WAKE	54.2 ± 3.5	**64.5 ± 4.6***	78.6 ± 6.3	75.4 ± 7.8
% SWS	37.4 ± 3.8	**27.5 ± 3.4***	17.2 ± 4.8	23.0 ± 3.9
% REM	8.3 ± 0.9	7.9 ± 2.6	4.2 ± 1.7	4.7 ± 1.1
% TST	45.3 ± 3.5	**35.4 ± 4.4***	21.4 ± 6.5	27.7 ± 4.9

B

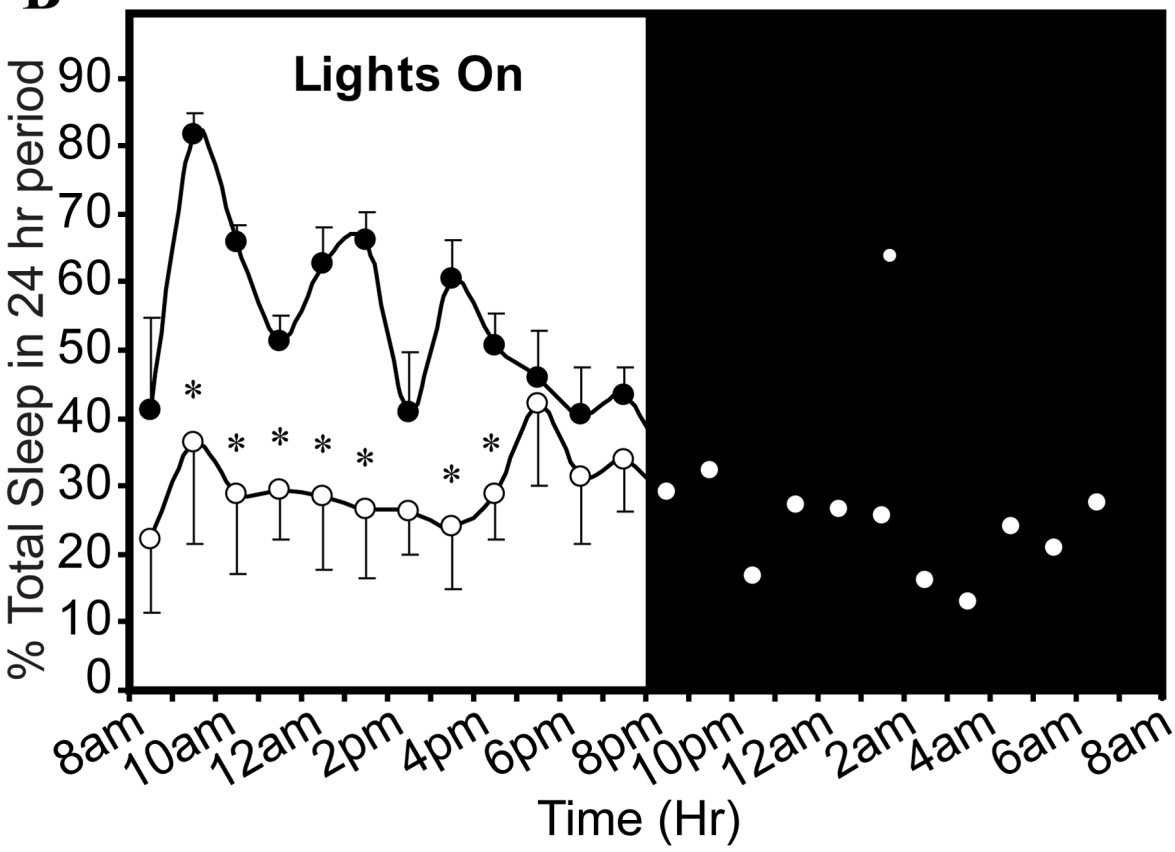

Figure 1.

Analysis of spontaneous sleep-wake behavior in young and old rats over a 24 hour period. The amount of SWS and total sleep time (%TST) were decreased during the lights-on period in aged rats compared young rats (*$p<0.05$). No significant differences were observed between age groups during the lights-off period. The percentages of Wake, SWS, and REM sleep in young rats over 24 h were comparable to previous reports (Tobler and Borbely, 1986).

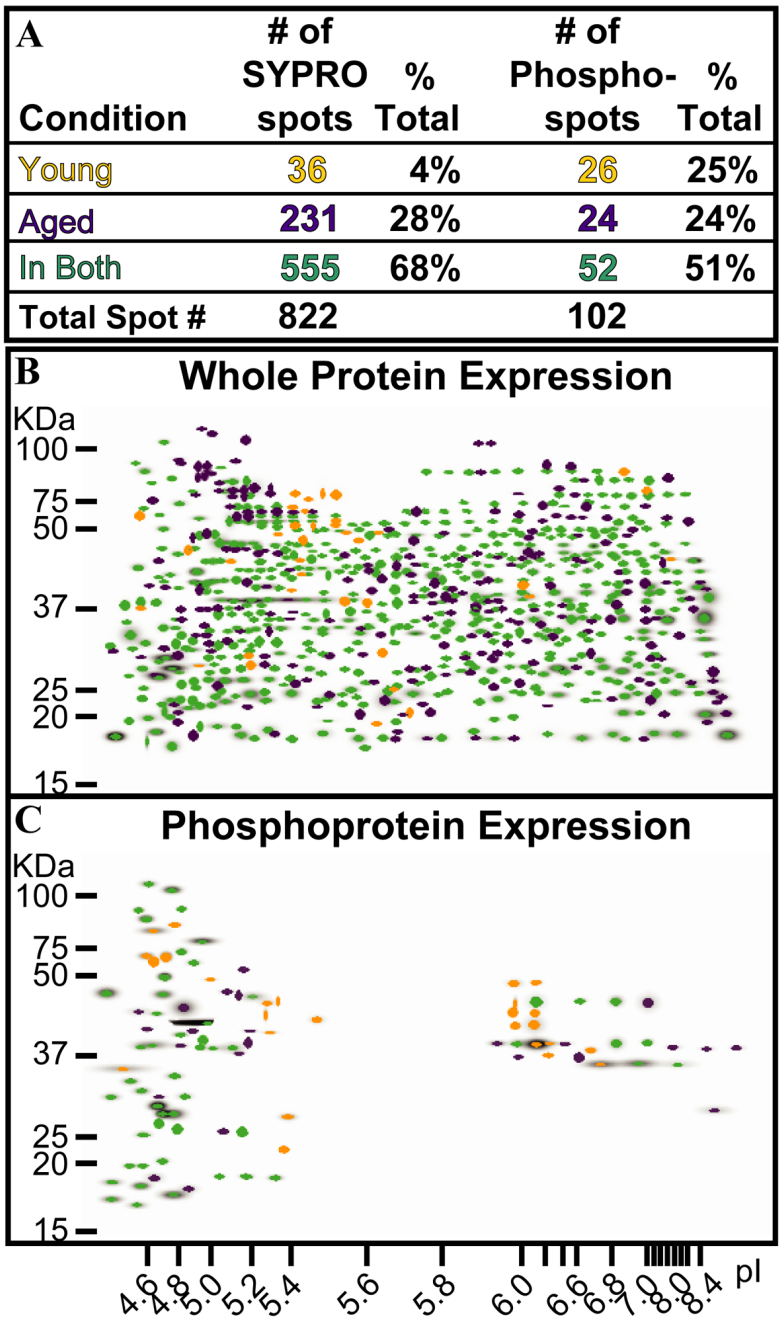

Figure 2.

Total and phosphoprotein analyses of young and old rats as a function of age. **(A).** Numerical analyses of SYPRO Ruby and ProQ Diamond profiles. (**B**). In the SYPRO Ruby Master image, spots are colored-coded to denote age-related protein expression. (**C**). ProQ Master image shows phosphoprotein spots expressed as a function of age. Green spots were present independent of age; purple spots; found only in old animals; gold spots; present only in young rats.

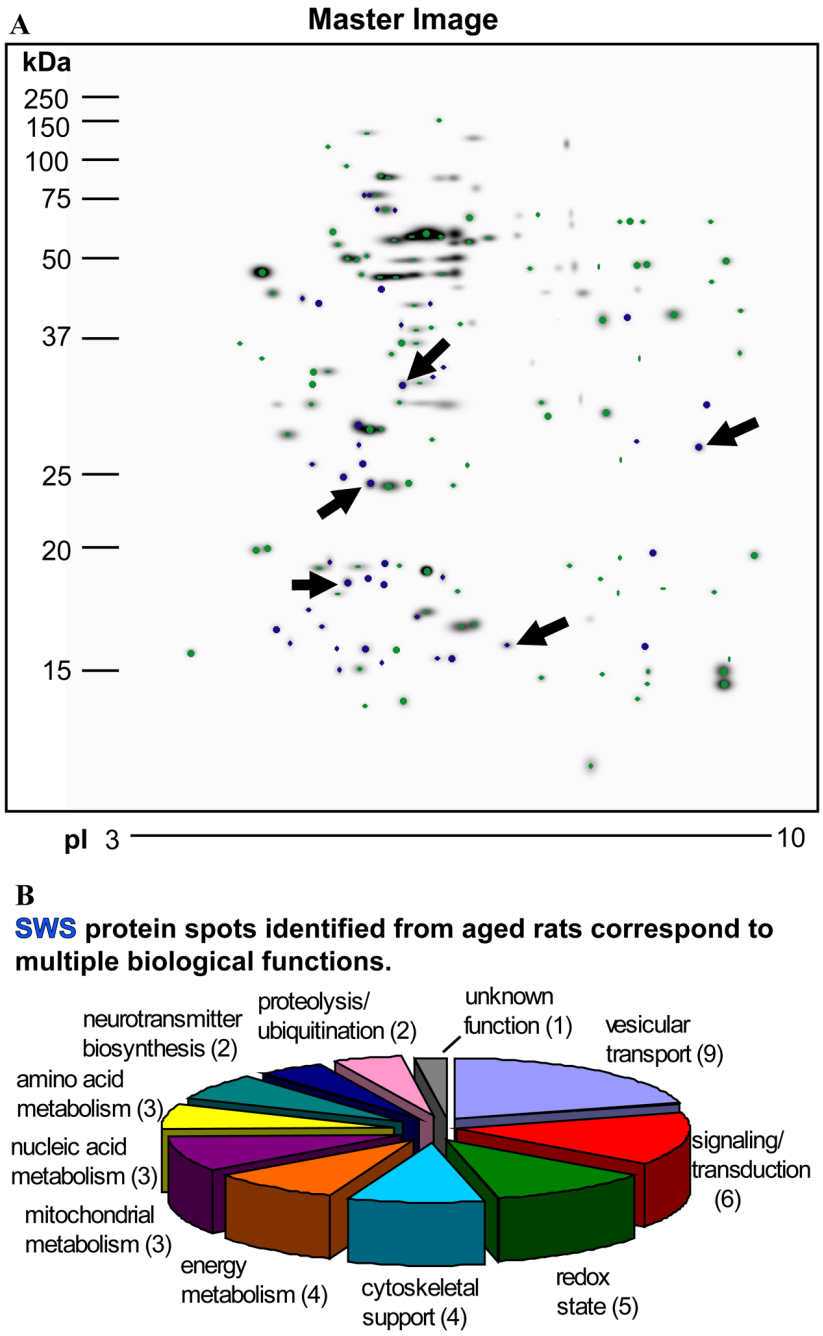

Figure 3.

Identification of protein spots in aged rats. **(A).** Large polyacrylamide gels were matched and analyzed according to age and behavioral state. Green spots indicate proteins common to both age (young and old) and behavioral state (W and SWS). Blue spots represent proteins expressed only in aged rats during SWS. Five of these spots (denoted by arrows) were chosen for MS analyses and identification. **(B).** SWS-related spots in aged animals were analyzed by mass spectroscopy. Proteins were associated with a variety of biological functions including vesicular transport, neurotransmitter synthesis, cytoskeletal support, energy metabolism, proteolysis, and the redox state of the cell. SWS-specific spots (shown in blue, panel A) identified in aged animals were associated with a variety of biological functions ranging from

vesicular transport, neurotransmitter synthesis, cytoskeletal support, energy metabolism, proteolysis, and the redox state of the cell. Of note, when a protein had known multiple classes of functions, it was assigned to the category that was best known based on the literature.

Table 1

Numerical analyses of SYPRO Ruby and ProQ Diamond protein expression as a function age and of behavioral state (s).

A	SYPRO Ruby				Phosphoprotein			
	YOUNG %		AGED %		YOUNG %		AGED %	
State	(n)	Total	(n)	Total	(n)	Total	(n)	Total
Wake	6	1%	30	4%	0	–	2	3%
SWS	12	2%	59	8%	21	27%	9	12%
In Both	18	3%	142	18%	5	6%	13	17%
Total #	591		786		78		76	

Table 2

Proteins identified from SWS- and age-unique selected 2DE gel spots.
Mass spectroscopy dentification of proteins from SWS- and age-unique selected 2DE gel spots.

ID #	Accession|Entry name	Protein name	Theor MW (Da)	Theor pI
1	P84079|ARF1_RAT	ADP-ribosylation factor 1	20697	6.31
2	Q66HA6|ARL8B_RAT	ADP-ribosylation factor-like protein 8B	21539	8.67
3	P37377|SYUA_RAT	Alpha-synuclein	14515	4.74
4	P27139|CAH2_RAT	Carbonic anhydrase 2 (EC 4.2.1.1)	28983	6.88
5	Q6P4Z9|CSN8_RAT	COP9 signalosome complex subunit 8	23236	5.09
6	P11348|DHPR_RAT	Dihydropteridine reductase (EC 1.5.1.34)	25552	7.67
7	Q6MG60|Q6MG60_RAT	Dimethylarginine dimethylaminohydrolase 2	29688	5.66
8	Q3T1J1|IF5A1_RAT	Eukaryotic translation initiation factor 5A-1	16832	5.07
9	P08009|GSTM4_RAT	Glutathione S-transferase Yb-3 (EC 2.5.1.18)	25681	6.84
10	Q71RR7|Q71RR7_RAT	Guanylate kinase (EC 2.7.4.8)	21910	5.44
11	P01946|HBA_RAT	Hemoglobin subunit alpha-1/2	15329	7.81
12	P62959|HINT1_RAT	Histidine triad nucleotide-binding protein 1	13779	6.64
13	P02262|H2A1_RAT	Histone H2A	14077	10.90
14	P62804|H4_RAT	Histone H4	11367	11.36
15	Q6P7Q4|LGUL_RAT	Lactoylglutathione lyase (EC 4.4.1.5)	20820	5.12
16	P02688|MBP_RAT	Myelin basic protein S	21502	11.24
17	Q9DCT2|NDUS3_MOUSE[1]	NADH dehydrogenase iron-sulfur protein 3, mitochondrial (EC 1.6.5.3)	30149	6.67
18	P08523|OMP_RAT	Olfactory marker protein	18853	5.12
19	Q63716|PRDX1_RAT	Peroxiredoxin-1 (EC 1.11.1.15)	22109	8.27
20	P35704|PRDX2_RAT	Peroxiredoxin-2 (EC 1.11.1.15)	21784	5.34
21	P31044|PEBP1_RAT	Phosphatidylethanolamine-binding protein 1	20801	5.47
22	P25113|PGAM1_RAT	Phosphoglycerate mutase 1 (EC 5.4.2.1) (EC 5.4.2.4) (EC 3.1.3.13)	28832	6.67
23	Q5M819|SERB_RAT	Phosphoserine phosphatase (EC 3.1.3.3)	24967	5.49
24	O35264|PA1B2_RAT	Platelet-activating factor acetylhydrolase 1B, subunit beta (EC 3.1.1.47)	25581	5.57
25	Q9EPC6|PROF2_RAT	Profilin-2	15002	6.55
26	P67779|PHB_RAT	Prohibitin	29820	5.57
27	Q6P9V7|Q6P9V7_RAT	Proteasome (Prosome, macropain) 28 subunit, alpha	28635	5.63
28	O35509|RB11B_RAT	Ras-related protein Rab-11B	24488	5.64
29	Q5EB77|RAB18_RAT	Ras-related protein Rab-18	22976	5.11
30	P05712|RAB2A_RAT	Ras-related protein Rab-2A	23535	6.08
31	P63012|RAB3A_RAT	Ras-related protein Rab-3A	24970	4.85
32	Q62636|RAB1B_RAT	Ras-related protein Rap-1B [precursor]	20798	5.65
33	P07632|SODC_RAT	Superoxide dismutase [Mn] (EC 1.15.1.1)	15912	5.88
34	P01830|THY1_RAT	Thy-1 membrane glycoprotein [Precursor]	18172	9.42
35	Q6PCT3|TPD54_RAT	Tumor protein D54	23992	5.80
36	Q498R7|CA123_RAT	Uncharacterized protein C1orf123 homolog	18095	5.06
37	P63045|VAMP2_RAT	Vesicle-associated membrane protein 2	12691	7.84
38	P62762|VISL1_RAT	Visinin-like protein 1	22142	5.01
39	P35213|1433B_RAT	14-3-3 protein beta/alpha	28054	4.81
40	P68255|1433T_RAT	14-3-3 protein theta	27778	4.69
41	P63102|1433Z_RAT	14-3-3 protein zeta/delta	27771	4.73
42	Q9CQ60|6PGL_MOUSE[1]	6-phosphogluconolactonase (EC 3.1.1.31)	27254	5.55

Legend

[1] Search engine reported mouse species.

Blast analysis (see methods) was performed on confidently identified peptide sequences to identify equivalent rodent species.